We confirmed microscopically that the tumor specimens were predominantly (>50%, on a nuclear basis) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use.

For immunohistochemical analysis, we used formalin-fixed and paraffin-embedded archival tissues from 116 patients with GC who underwent surgery between 1975 and 2005 at the Department of Surgical Oncology, Hiroshima University Hospital. All 116 patients were A-bomb survivors (LSS cohort members) in Hiroshima, Japan. Although these patients were survivors who developed GC after the bombing, they were further classified according to the levels of exposed radiation dose (*i.e.* ≥5 mGy and <5 mGy were defined as "exposed" and "nonexposed," respectively): 64 exposed (median dose, 51 mGy) and 52 nonexposed patients.

Tumor staging was performed according to the TNM classification system.[21] Histologic classification of GC was carried out according to the Lauren classification system.[22] The detailed procedures for acquiring informed consent from study patients and collecting tissue specimens were as described previously.[23] In accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used on the basis of the approval from the Ethical Review Committee of the Hiroshima University School of Medicine and from the ethical review committees of collaborating organizations.

Radiation dose

A-bomb radiation doses were estimated with the DS02 system.[24]

Oligonucleotide array construction

Probes, which were all 65 bp in length, were designed to have approximately the same annealing temperature. The oligonucleotide array, Genopal™ (Mitsubishi Rayon, Tokyo, Japan), was made as described previously.[25] In brief, plastic hollow fibers were bundled in an orderly arrangement and hardened with resin to form a block. Oligonucleotide-capture probes were chemically bonded inside each hollow fiber with hydrophilic gel. The block was then sliced into thin chips, each of which was set in a holder (www.mrc.co.jp/genome/about/process.html for details). The array contained 208 genes, including GC-related genes identified by our previous SAGE analysis,[26] known genes related to development and progression of GC,[27,28] genes related to DNA damage response and repair and genes associated with sensitivity to anticancer drugs.[29] A list of the genes on the array is available upon request.

Preparation of labeled probe, hybridization, detection and data analysis

Total RNA was isolated from frozen tissue with Isogene (Nippon Gene, Tokyo, Japan), according to the manufacturer's protocol. Quantification and integrity of RNA were assessed with an Agilent 2100 Bioanalyzer and RNA 6000 LabChip Kit (Agilent Technologies, Palo Alto, CA). One microgram of total RNA was used to prepare antisense biotinylated RNA with MessageAmp™ II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion, Austin, TX) per the manufacturer's instructions. The biotinylated cRNAs were then cleaned up and fragmented. Hybridization was carried out with the oligonucleotide array in 100 μl of hybridization buffer (0.12 M Tris HCl/0.12 M NaCl/0.05% Tween-20 and 5 μg of fragmented biotinylated aRNA) at 65°C overnight. After hybridization, the oligonucleotide arrays were washed twice in 0.12 M Tris HCl/0.12 M NaCl/0.05% Tween-20 at 65°C for 20 min, followed by washing in 0.12 M Tris HCl/0.12 M NaCl for 10 min, before being cooled slowly to room temperature. After staining with streptavidin–Alexa Fluor 647 (Invitrogen, Carlsbad, CA), the Genopal array was scanned, and the image was captured with a cooled CCD-type Microarray Image Analyzer (Mitsubishi Rayon). Fluorescence intensity was analyzed with software developed by Mitsubishi Rayon. Fluorescence throughout the 3-dimensional structure of each array feature can be efficiently captured because of the long focal depth of the optical system of the image analyzer (www.mrc.co.jp/genome/about/analysis.html). After image acquisition and quantification, spots with signal intensity lower than or equal to that of the background were identified and excluded from the analysis. Next, background-subtracted spot intensities were normalized so that the *ACTB* gene signal would be 10,000.

Immunohistochemistry

Formalin-fixed and paraffin-embedded samples were sectioned, deparaffinized and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically with a Dako Envision + Rabbit Peroxidase Detection System (Dako Cytomation, Carpinteria, CA) or Dako Envision + Mouse Peroxidase Detection System (Dako Cytomation). Antigen retrieval was done by microwave heating in citrate buffer (pH 6.0) for 30 min. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block nonspecific antibody binding sites. Sections were incubated with primary antibodies against transketolase (dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), versican (1:50; Seikagaku Corporation, Tokyo, Japan), THBS-2 (1:50; Santa Cruz Biotechnology), PDGF receptor-β (1:50; Santa Cruz Biotechnology), ribonuclease A (1:50; Abcam, Cambridge, UK), osteonectin (1:50; Novocastra, Newcastle, UK), vimentin (1:50, Dako Cytomation) and Ki67 (1:50, Dako Cytomation) for 1 hr at room temperature, followed by incubations with Envision + antirabbit peroxidase or Envision + antimouse peroxidase for 30 min each. Staining was completed with 10 min incubation with the substrate–chromogen solution. Sections were counterstained with 0.1% hematoxylin. For the Ki67-index, 1,000 nuclei were counted to evaluate the percentage of positive nuclei. The Ki67-index was considered to reflect the proliferative index.

Phenotype analysis of GC

GCs were classified into 4 phenotypes: gastric (G) type, intestinal (I) type, gastric and intestinal mixed (GI) type and unclassified (N) type. For phenotypic expression analysis of GC, we performed immunohistochemical analysis (as described earlier) with 4 antibodies: anti-MUC5AC (Novocastra) as a marker of gastric foveolar epithelial cells, anti-MUC6 (Novocastra) as a marker of pyloric gland cells, anti-MUC2 (Novocastra) as a marker of goblet cells in the small intestine and colorectum, and anti-CD10 (Novocastra) as a marker of microvilli of absorptive cells in the small intestine and colorectum. The criteria[30] for the classification of G-type and I-type GCs were as follows. GCs in which more than 10% of cells in the section expressed at least 1 gastric epithelial cell marker (MUC5AC or MUC6) or intestinal epithelial cell marker (MUC2 or CD10) were classified as G-type or I-type cancers, respectively. Sections that showed both gastric and intestinal phenotypes were classified as GI type, and those that lacked both the gastric and intestinal phenotypes were classified as N type.

Statistical methods

Correlation between the gene expression profiles from different sample conditions was assessed by Spearman's rank correlation coefficients. Differences in mRNA expression levels between 2 samples were tested by Mann–Whitney *U* test for individual genes. Univariate analysis for clinicopathologic, phenotypic and proliferative variables in relation to radiation exposure status was done by Mann–Whitney *U* test for continuous variables and Fisher's exact test for categorical variables. Associations between clinicopathologic variables and immunostaining for versican or osteonectin were analyzed by Fisher's exact test. Multivariate logistic regression analysis was carried out to assess the relationship among clinicopathologic characteristics, expression of versican

and osteonectin, and radiation exposure status. A p value of <0.05 was considered statistically significant.

Results

Custom array analysis

Toward identification of potential molecular markers for radiation-associated cancer and also better understanding of its molecular mechanisms, we designed a custom oligonucleotide array comprising 208 genes (Fig. 1). Because this platform has not been characterized, we first validated the performance of this array before analyzing the GCs from exposed patients. To validate the array, total RNA was isolated from the MKN-1 cell line. RNA quality was assessed with a Bioanalyzer (Agilent), and the RNA integrity number (RIN) was confirmed to be 10.0. The isolated RNA was divided into 5 tubes (Samples 1–5), and we subjected the RNA in each tube to a different preparation condition. To analyze the effect of the amount of total RNA, we prepared 3 samples. Sample 1 contained 1.0 μg total RNA, Sample 2 contained 2.0 μg total RNA and Sample 3 contained 0.5 μg total RNA. To analyze the effect of RNA quality, we prepared 2 RNA samples with different RINs. Sample 4 was frozen and thawed 20 times, and Sample 5 was frozen and thawed 40 times. The RNA quality was then assessed with the Bioanalyzer. The RIN of Sample 4 was 8.2 and that of Sample 5 was 5.9. Probes derived from these 5 samples were hybridized simultaneously (Fig. 1). Although the condition of each sample differed, gene expression levels from Sample 1 obtained by oligonucleotide array correlated well with those from Sample 2 ($p < 0.0001$, $r = 0.95$), Sample 3 ($p < 0.0001$, $r = 0.96$), Sample 4 ($p < 0.0001$, $r = 0.98$) and Sample 5 ($p < 0.0001$, $r = 0.97$) (Fig. 1). Therefore, 1 μg of total RNA (RIN > 6.0) was used for further array analysis.

We next analyzed the gene expression profiles of GCs from exposed and nonexposed patients by custom oligonucleotide array. Freshly frozen GC tissue samples were obtained from 3 exposed patients (Cases EX01, EX02 and EX03, with radiation dose 7, 5 and 18 mGy, respectively) as well as their corresponding nonneoplastic mucosa samples. Clinicopathologic features of these exposed patients are shown in Table I. Histologically, EX01 was intestinal-type GC of Lauren classification, EX02 was diffuse-type GC of Lauren classification, and EX03 was an α-fetoprotein (AFP)-producing hepatoid adenocarcinoma (Fig. 2*a*). Immunostaining of EX03 revealed that AFP was present in cancer cells (Fig. 2*a*). On the other hand, when we analyzed 20 freshly frozen GC tissues from nonexposed patients (Cases NEX01 to NEX20), unsupervised clustering showed that the exposed and nonexposed patients could not be distinguished on the basis of mRNA expression (data not shown). To determine whether there is a gene expression profile characteristic to exposed patients, we compared expression levels of individual genes between exposed and nonexposed patients. Finally, we found 9 genes whose expression was significantly lower in GC from exposed patients than in GC from nonexposed patients (Table II). No gene showed higher expression in GC from exposed patients than in GC from nonexposed patients.

Among these 9 genes, antibodies against proteins encoded by *TKT* (encoding transketolase), *VCAN* (encoding versican), *THBS2* (encoding THBS-2), *PDGFRB* (encoding PDGF receptor-β), *RNASE1* (encoding ribonuclease A) and *SPARC* (encoding osteonectin) were commercially available. We performed immunohistochemistry of these 6 molecules in 3 formalin-fixed and paraffin-embedded archival GC tissue samples from exposed patients and 10 GC samples from nonexposed patients analyzed by oligonucleotide array to compare oligonucleotide array data and immunostaining results. The immunostaining results for versican (encoded by *VCAN*) and osteonectin (encoded by *SPARC*) were consistent with those of the oligonucleotide array. It has been reported that immunoreactivity for versican was present in tumor-stroma associated with malignant areas and in blood vessel walls.[31] In Case EX03 (exposed patient), expression of versican (*VCAN*) mRNA

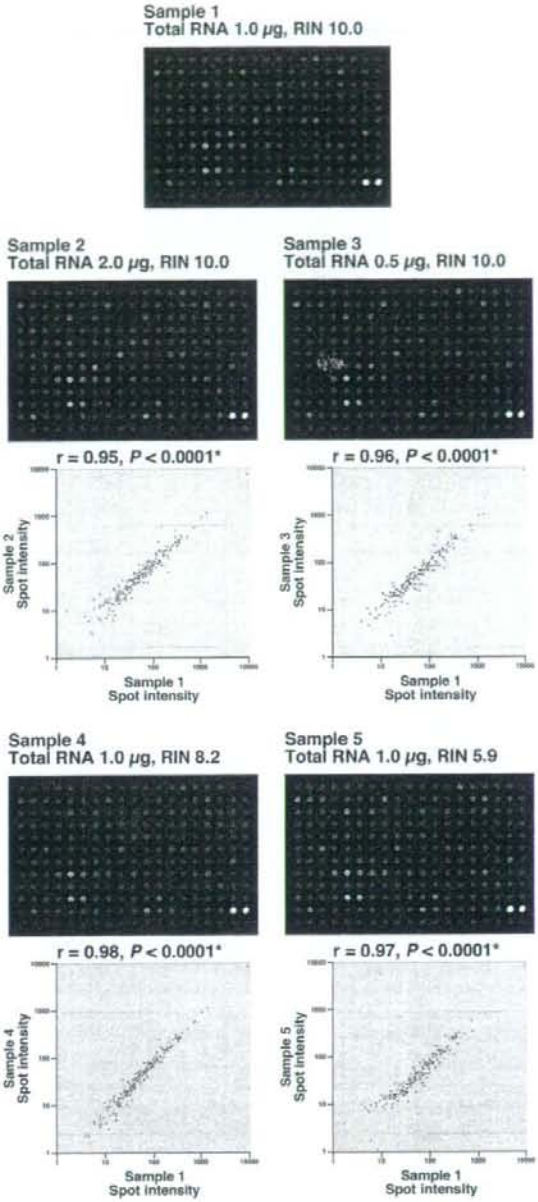

FIGURE 1 – Custom oligonucleotide array analysis of GCs from exposed and nonexposed patients. Images of hybridization results for probes prepared under 5 different conditions. The oligonucleotide arrays' intensity data for Samples 1 and 2, Samples 1 and 3, Samples 1 and 4 and Samples 1 and 5 are plotted against each other. *Spearman's rank correlation coefficient.

on the oligonucleotide array was low, and immunohistochemistry revealed that tumor-associated stroma did not express versican despite staining of versican in blood vessel walls (Fig. 2*b*). In Case EX01 and Case EX02, versican staining was not observed. In Case NEX03 (nonexposed patient), strong and extensive staining

TABLE I – CLINICOPATHOLOGIC CHARACTERISTICS OF THE 3 GASTRIC CANCERS FROM EXPOSED PATIENTS ANALYZED BY OLIGONUCLEOTIDE ARRAY

Sample name	Age (years)	Sex	T grade	N grade	M grade	Stage	Histologic classification	Radiation dose (mGy)
EX01	72	Female	2	0	0	I	Intestinal	7
EX02	75	Male	2	1	0	II	Diffuse	5
EX03	62	Female	2	1	0	II	Hepatoid	18

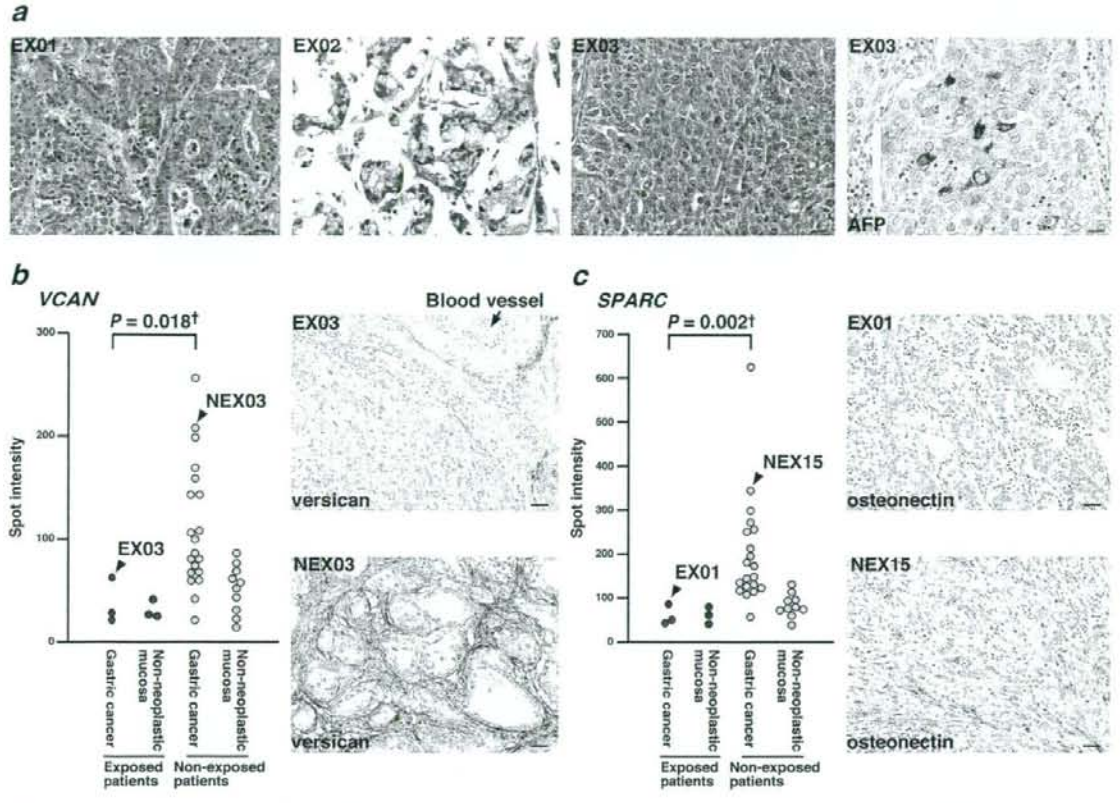

FIGURE 2 – (*a*) H&E-stained sections of GCs from exposed patients analyzed by custom oligonucleotide array. Case EX01 was an intestinal-type GC of Lauren classification, and Case EX02 was a diffuse-type GC of Lauren classification. EX03 was an AFP-producing hepatoid adenocarcinoma. Staining of AFP was observed in tumor cells (brown color). Bar: 25 μm. (*b*) Analyses of expression of *VCAN* mRNA (which encodes versican) by custom oligonucleotide array (left) and expression of versican protein by immunohistochemistry (right) in GCs from exposed and nonexposed patients. Each point represents the *VCAN* mRNA expression level. †Mann–Whitney *U* test. Immunostaining of versican in GC from exposed patients (Case EX03) and nonexposed patients (Case NEX03) revealed that tumor-associated stromal cells express versican. Versican staining in blood vessel wall was also observed (arrow). Bar: 50 μm. (*c*) Analyses of expression of *SPARC* mRNA (which encodes osteonectin) by custom oligonucleotide array (left) and expression of osteonectin protein by immunohistochemistry (right) in GCs from exposed and nonexposed patients. Each point represents the *SPARC* mRNA expression level. †Mann–Whitney *U* test. In GC from exposed patients (Case EX01) and nonexposed patients (Case NEX15), tumor-associated stromal cells express osteonectin. Bar: 50 μm.

of versican was observed in tumor-associated stroma (Fig. 2*b*). Stromal versican staining was also found in several GC cases from nonexposed patients. In addition to versican, osteonectin has also been reported to be stained in tumor-associated stroma. In Case EX01 (exposed patient), expression of osteonectin (*SPARC*) mRNA on the oligonucleotide array was low, and a small fraction of tumor-associated stromal cells was shown to express osteonectin by immunohistochemistry (Fig. 2*c*). In Case EX02 and Case EX03, osteonectin staining was not observed. In contrast, in Case NEX15 (nonexposed patient), extensive staining of osteonectin was observed in tumor-associated stroma (Fig. 2*c*). Stromal osteonectin staining was also found in several GC cases from nonexposed patients. Immunostaining of the remaining 4 molecules (transketolase, THBS-2, PDGF receptor-β and ribonuclease A) did not differ significantly between exposed and nonexposed patients. Therefore, we decided to perform immunostaining of versican and osteonectin in additional GC cases.

Clinicopathologic features, mucin phenotypes and proliferative characteristics of survivor patients

To validate the reduced expression of versican and osteonectin in GC from exposed patients, we collected formalin-fixed and paraffin-embedded archival tissues from 116 patients with GC who underwent surgical resection. All 116 patients were A-bomb survivors (LSS cohort members) in Hiroshima, Japan, and comprised

64 exposed (median dose, 51 mGy; range, 5–2,601 mGy) and 52 nonexposed patients (range, 0–4 mGy) who developed GC after the bombing. Patient characteristics such as latency period (years elapsed from A-bombing to diagnosis, defined only for exposed patients), age at the time of A-bombing, histologic type, sex, age at diagnosis, T grade, N grade and tumor stage are summarized in Table III. Clinicopathologic characteristics of patients did not statistically differ between exposed and nonexposed patients. Diffuse-type GC was found more frequently in exposed patients than in nonexposed patients, but the difference was not statistically significant ($p = 0.085$).

Despite the usefulness of the Lauren classification, it was previously reported that GC can be subdivided according to mucin expression into 4 phenotypes (G type, I type, GI type and N type).[32] Several distinct genetic and epigenetic changes have been reported to be associated with G-type and I-type GCs.[33–35] Therefore, we investigated the mucin phenotypes of 116 GCs: Gastric (MUC5AC and MUC6) and intestinal (MUC2 and CD10). MUC5AC was detected in 57 of 116 (49%) cases, MUC6 in 37 (32%) cases, MUC2 in 34 (29%) cases and CD10 in 25 (22%) cases. Expression of these 4 markers did not differ statistically between exposed and nonexposed patients (data not shown). In addition, distribution of the G, I, GI and N phenotypes did not differ significantly between exposed and nonexposed patients (data not shown).

Phenotypic shift from G-type to I-type GC along with tumor progression has been reported.[32] Therefore, mucin phenotypes and immunostaining of MUC5AC, MUC6, MUC2 and CD10 were analyzed with respect to tumor stages. In Stage I/II, expression of the 4 markers did not differ significantly between exposed and nonexposed patients (data not shown). In contrast, in Stage III/IV, some GCs from exposed patients showed extensive staining of MUC6 (Fig. 3*a*). In addition, staining of MUC2 was rare in GCs from exposed patients (Fig. 3*a*). Of 28 Stage III/IV GCs from exposed patients, MUC6 was expressed in 12 GCs (43%), whereas MUC6 was expressed in 2 (12%) of 17 Stage III/IV GCs from nonexposed patients ($p = 0.046$). In Stage III/IV cases, the frequency of MUC2 expression in GCs from exposed patients (3/28, 11%) was significantly lower than that in GCs from nonexposed patients (8/17, 47%, $p = 0.011$). There was no correlation between exposure status and MUC5AC or CD10 (data not shown). Mucin phenotypes with respect to tumor stage are shown in Figure 3*b*. For Stage I/II GCs, distribution of the G, I, GI and N types did not differ significantly between exposed and nonexposed patients. Among GCs from nonexposed patients, frequency of the G type slightly decreased with advancing tumor stage. In contrast, among GCs from exposed patients, frequency of the G type increased with advancing tumor stage: furthermore, frequency of the I type decreased with advancing tumor stage. However, statistical analysis showed that neither frequency of the G type (Table IV) nor I type (Table V) differed between exposed and nonexposed patients.

Proliferative characteristics of GCs from exposed and nonexposed patients were also investigated. The Ki67-index did not differ significantly between exposed and nonexposed patients (Table III). This was also the case in Stage I/II (data not shown). These results indicate that no significant differences in clinicopathologic, phenotypic and proliferative characteristics of GCs were found between exposed and nonexposed patients, at least in Stage I/II GCs.

TABLE II – NINE GENES WITH LOWER EXPRESSION IN GASTRIC CANCERS FROM EXPOSED PATIENTS THAN IN GASTRIC CANCERS FROM NONEXPOSED PATIENTS

Gene symbol	Exposure status	mRNA expression: median (range)	p value[1]
SPARC	Exposed	46 (39–84)	0.002
	Nonexposed	153 (55–624)	
ABCC1	Exposed	19 (15–22)	0.002
	Nonexposed	43.5 (19–101)	
RNASE1	Exposed	75 (74–100)	0.012
	Nonexposed	425 (46–1632)	
VCAN	Exposed	29 (24–63)	0.018
	Nonexposed	92 (22–258)	
THBS2	Exposed	35 (19–81)	0.018
	Nonexposed	155.5 (39–411)	
PDGFRB	Exposed	20 (18–29)	0.018
	Nonexposed	49.5 (13–138)	
TNFRSF14	Exposed	27 (25–42)	0.035
	Nonexposed	49 (29–90)	
TKT	Exposed	58 (35–100)	0.035
	Nonexposed	128 (53–253)	
FBP1	Exposed	105 (36–114)	0.046
	Nonexposed	196.5 (55–509)	

[1]Mann–Whitney U test.

TABLE III – CLINICOPATHOLOGIC CHARACTERISTICS OF PATIENTS BY RADIATION EXPOSURE STATUS

Variable	Exposed patients ($n = 64$)	Nonexposed patients ($n = 52$)	p value
Median radiation dose (mGy, range)	51 (5–2601)	0 (0–4)	
Median latency period (years, range)	47 (30–60)	46 (30–57)	0.5[1]
Median age at the time of atomic-bombing (years, range)	25 (2–47)	28 (2–47)	0.098[1]
Sex			
Male	34	27	1.0[2]
Female	30	25	
Age at diagnosis (years)			
≤65	18	12	0.7[2]
>65	46	40	
T grade			
T1/T2	43	35	1.0[2]
T3/T4	21	17	
N grade			
N0	24	28	0.093[2]
N1/N2/N3	40	24	
Stage			
I/II	36	35	0.3[2]
III/IV	28	17	
Lauren classification			
Intestinal	35	37	0.085[2]
Diffuse	29	15	
Median Ki67-index (%, range)	33 (11–69)	39 (10–64)	0.196[1]

[1]Mann–Whitney U test.–[2]Fisher's exact test.

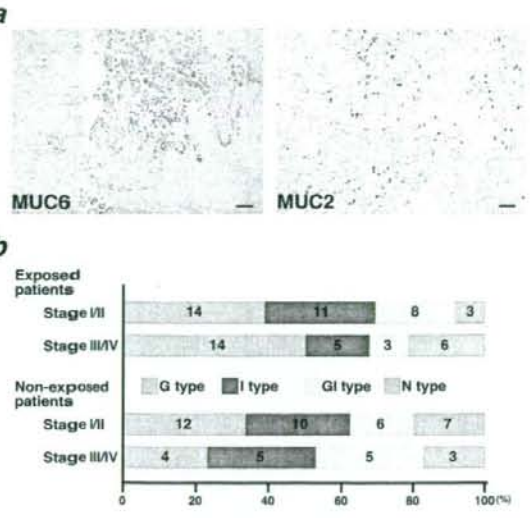

FIGURE 3 – Phenotypic analysis of GCs from exposed and nonexposed patients. (*a*) Immunostaining of MUC6 (exposed patient) and MUC2 (nonexposed patient). Bar: MUC6, 250 μm; MUC2, 100 μm. (*b*) Distribution of G, I, GI and N-type GCs from exposed and nonexposed patients. Numbers indicate the number of cases.

TABLE IV – SUMMARY OF THE FREQUENCY OF G-TYPE GASTRIC CANCERS IN STAGE III/IV FROM EXPOSED AND NONEXPOSED PATIENTS

	G type	Other type	p value[1]
Exposed patients	14 (50%)	14	0.118
Nonexposed patients	4 (24%)	13	

[1]Fisher's exact test.

Decreased expression of versican and osteonectin in exposed patients

We performed immunostaining of versican and osteonectin in 116 GCs. In nonneoplastic gastric mucosa, although epithelial cells and stromal cells exhibited weak or no expression of versican (Figs. 4*a* and 4*b*), versican staining was observed in the walls of blood vessels (Fig. 4*c*). In GC tissue, tumor cells were not stained; however, versican was expressed in tumor-associated stroma. Intracellular staining of versican was detected in fibroblastic cells, because of their morphology and vimentin (a marker of fibroblasts) positivity on serial sections. Staining in fibrous bands within the tumor was also observed. The level of versican immunoreactivity was evaluated in the area of tumor-associated stroma (fibroblastic cell plus ECM). The percentage of versican-stained area of tumor-associated stroma was a continuum from 0 to 80%, and characteristic staining pattern was not observed. To analyze the relationship of versican staining to clinicopathologic characteristics, the GC cases were divided into two groups: diffuse positive group and focal positive or negative group. To maximize the statistical detection power, it is ideal that the number of diffuse positive cases/focal positive or negative cases ratio is less than 2 in nonexposed patients. When we chose 50% (50% of the area of tumor-associated stroma) as a cutoff for diffuse positive group and focal positive or negative group, 32 (62%) were diffuse positive for versican in 52 GC cases from nonexposed patients. Therefore, the immunostaining was considered diffuse positive for versican when more than 50% of the area of tumor-associated stroma was stained by versican. Of 116 GC cases, 55 (47%) were diffuse positive for versican. Figure 4*d* illustrates the typically diffuse positive immunostaining of versican. In diffuse-type GC, strong and extensive stromal versican staining was frequently observed (Fig. 4*d*). Staining in fibrous bands within the tumor was also observed. Figure 4*e* illustrates the typically focal positive immunostaining of versican. In many GCs from exposed patients, although blood vessel wall expressed versican, staining of versican was weak or absent in stromal fibroblasts and stromal matrix (Fig. 4*e*). Diffuse positive GCs correlated to advanced T grade ($p = 0.003$), N grade ($p = 0.016$) and tumor stage ($p = 0.001$) (Table VI). Diffuse positive GCs were more frequently found in diffuse-type GCs than in intestinal-type GCs ($p = 0.008$) (Table 6). Focal positive or negative GCs were more frequently found in exposed patients than in nonexposed patients ($p = 0.009$) (Table 6). Because expression of versican correlated with tumor stage, versican immunostaining was analyzed by tumor stages. As shown in Table 6, in both Stage I/II and Stage III/IV GCs, focal positive or negative GCs were more frequently found in exposed patients than in nonexposed patients ($p = 0.026$ and $p = 0.023$, respectively). We then examined whether versican staining in GC from exposed patients was related to radiation dose, latency period or age at the time of A-bombing; however, there was no correlation between versican staining and any of these variables. Although versican staining was correlated with tumor stage in 116 GC cases ($p = 0.001$), multivariate logistic regression analysis revealed that expression of versican was not significant independent marker for tumor stage in 64 exposed patients ($p = 0.6$). Among 52 nonexposed patients, multivariate logistic regression analysis showed that expression of versican was not significant independent marker for tumor stage ($p = 0.9$).

TABLE V – SUMMARY OF THE FREQUENCY OF I-TYPE GASTRIC CANCERS IN STAGE III/IV FROM EXPOSED AND NONEXPOSED PATIENTS

	I type	Other type	p value[1]
Exposed patients	5 (18%)	23	0.5
Nonexposed patients	5 (29%)	12	

[1]Fisher's exact test.

We next investigated expression of osteonectin. In nonneoplastic mucosa, epithelial cells did not express osteonectin. However, in some cases, stromal cells exhibited osteonectin immunoreactivity (Fig. 4*f*). In GC tissues, osteonectin was stained in tumor-associated stroma; however, tumor cells were not stained. Intracellular staining of osteonectin was detected in fibroblastic cells because of their morphology and vimentin positivity on serial sections. As in a previous study,[20] immunoreactivity was also evident in the stromal matrix. Therefore, the levels of osteonectin immunoreactivity were evaluated in the area of tumor-associated stroma (fibroblastic cell plus ECM). The percentage of osteonectin-stained area of tumor-associated stroma ranged from 0 to 80%. Among 116 GCs, osteonectin-positive stromal cells were observed at the superficial parts of tumors in 44 (38%) cases (Fig. 4*g*). Osteonectin staining at the superficial parts of tumors was not correlated with T grade, N grade, tumor stage, histological type or exposure status (data not shown). To further analyze the relationship of osteonectin staining to clinicopathologic characteristics, the GC cases were divided into 2 groups: diffuse positive group and focal positive or negative group. When the same cutoff point for osteonectin and versican immunostaining was set (50% of the area of tumor-associated stroma), 20 (38%) were diffuse positive for osteonectin in 52 GC cases from nonexposed patients, and the number of diffuse positive cases/focal positive or negative cases ratio was less than 2 in nonexposed patients. Of 116 GC cases, 29 (25%) were diffuse positive for osteonectin. Figure 4*h* illustrates the typically diffuse positive immunostaining of osteonectin. Extensive staining of stromal cells was frequently observed in late-stage GC. Immunoreactivity was also evident in the stromal matrix. Figure 4*i* illustrates the typically focal positive or negative immunostaining of osteonectin. In many GCs from exposed patients, only few stromal

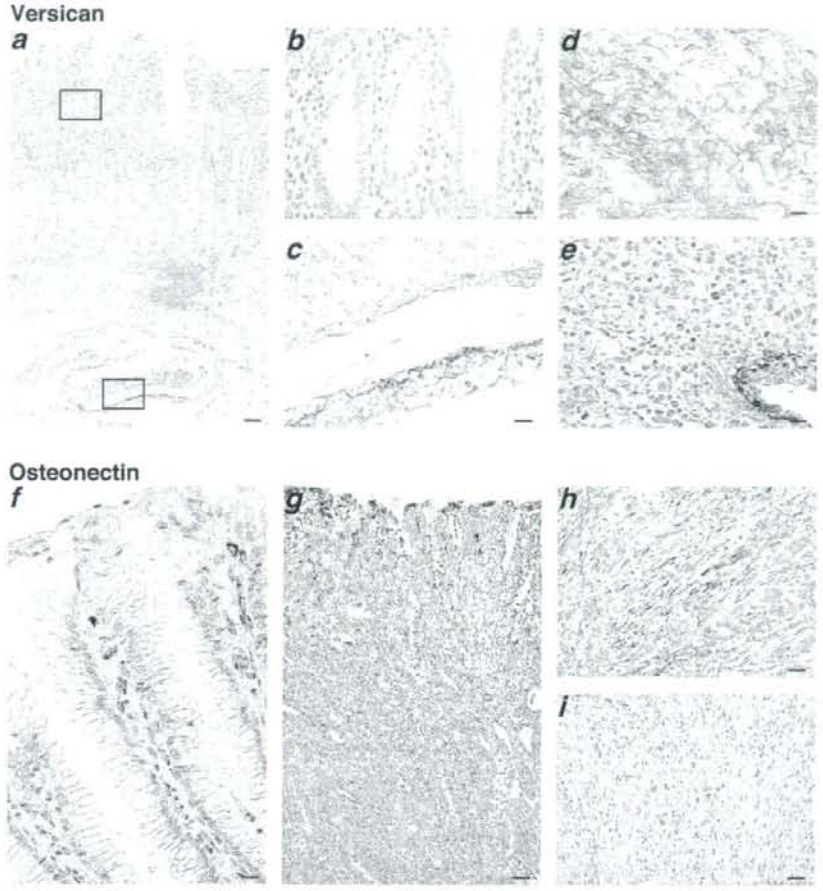

FIGURE 4 – (*a–e*) Immunohistochemical analysis of versican and osteonectin in 116 GCs from exposed and nonexposed patients. (*a*) Immunostaining of versican in GCs from exposed and nonexposed patients. (*a*) Nonneoplastic gastric mucosa. Panels (*b*) and (*c*) show high-magnification images of the fields indicated by boxes in panel (*a*). Bar: 150 μm. (*b*) Foveolar epithelium in nonneoplastic gastric mucosa is negative for versican. Bar: 25 μm. (*c*) Blood vessel wall in nonneoplastic gastric mucosa expresses versican. Bar: 25 μm. (*d*) Stromal cells in GC from nonexposed patients express versican. Bar: 25 μm. (*e*) Staining of versican was weak or absent in GCs from exposed patients. Bar: 25 μm. (*f–i*) Immunostaining of osteonectin in GCs from exposed and nonexposed patients. (*f*) Nonneoplastic gastric mucosa. Some stromal cells are positive for osteonectin. Bar: 12 μm. (*g*) In some GC cases, osteonectin-positive stromal cells are observed at the superficial part of the tumor tissue. Bar: 50 μm. (*h*) Stromal cells in GC from nonexposed patients are positive for osteonectin. Bar: 50 μm. (*i*) Few stromal cells are stained in GC from exposed patients. Bar: 50 μm.

cells exhibited osteonectin staining. Immunoreactivity was not found in the stromal matrix. Diffuse positive GCs correlated to advanced T grade ($p = 0.006$), N grade ($p = 0.011$) and tumor stage ($p = 0.004$) (Table VI). Focal positive or negative GCs were more frequently found in exposed patients than in nonexposed patients ($p = 0.005$) (Table VI). Because expression of osteonectin correlated with tumor stage, we analyzed osteonectin immunostaining by tumor stage. As shown in Table VI, in both Stage I/II and Stage III/IV GCs, focal positive or negative GCs were more frequently found in exposed patients than in nonexposed patients ($p = 0.024$ and $p = 0.013$, respectively). We then examined whether osteonectin staining in GCs from exposed patients was related to radiation dose, latency period or age at the time of A-bombing; however, there was no correlation between osteonectin staining and these variables. Although diffuse positive GCs correlated to tumor stage ($p = 0.004$), multivariate logistic regression analysis revealed that expression of osteonectin was not significant independent marker for tumor stage in 64 exposed patients ($p = 0.057$). Among 52 nonexposed patients, multivariate logistic regression analysis showed that expression of osteonectin was not significant independent marker for tumor stage ($p = 0.9$).

Because clinicopathologic characteristics and expression of versican and osteonectin may be interrelated, we performed multivariate logistic analysis to determine which variables are independent markers for radiation exposure status. As shown in Table VII, multivariate logistic regression analysis revealed that both expression of versican ($p = 0.002$) and osteonectin ($p = 0.001$) were significant independent markers for GCs from exposed patients.

Discussion

Our study entails 2-stage strategy to find molecular markers that are specifically involved in radiation-associated gastric carcinogenesis among A-bomb survivors. First, candidate genes were selected by custom oligonucleotide array, developed by us, which

TABLE VI – RELATION BETWEEN VERSICAN OR OSTEONECTIN EXPRESSION AND CLINICOPATHOLOGIC CHARACTERISTICS OF GASTRIC CANCER

Variable	Versican			Osteonectin		
	Diffuse positive (%)	Focal positive or negative	p value[1]	Diffuse positive (%)	Focal positive or negative	p value[1]
Sex						
Male	30 (49)	31	0.7	16 (26)	45	0.8
Female	25 (45)	30		13 (24)	42	
Age at diagnosis						
≤65	15 (50)	15	0.8	9 (30)	21	0.5
>65	40 (47)	46		20 (23)	66	
T grade						
T1/T2	29 (37)	49	0.003	13 (17)	65	0.006
T3/T4	26 (68)	12		16 (42)	22	
N grade						
N0	18 (35)	34	0.016	7 (13)	45	0.011
N1/N2/N3	37 (58)	27		22 (34)	42	
Stage						
I/II	25 (35)	46	0.001	11 (15)	60	0.004
III/IV	30 (67)	15		18 (40)	27	
Lauren classification						
Intestinal	27 (38)	45	0.008	17 (24)	55	0.7
Diffuse	28 (64)	16		12 (27)	32	
Exposure status (all cases)						
Exposed	23 (36)	41	0.009	9 (14)	55	0.005
Nonexposed	32 (62)	20		20 (38)	32	
Exposure status (Stage I/II cases)						
Exposed	8 (22)	28	0.026	2 (6)	34	0.024
Nonexposed	17 (49)	18		9 (26)	26	
Exposure status (Stage III/IV cases)						
Exposed	15 (54)	13	0.023	7 (25)	21	0.013
Nonexposed	15 (88)	2		11 (65)	6	
Exposure status (intestinal type of Lauren classification)						
Exposed	9 (26)	26	0.054	5 (25)	30	0.097
Nonexposed	18 (49)	19		12 (65)	25	
Exposure status (diffuse type of Lauren classification)						
Exposed	14 (48)	15	0.003	4 (14)	25	0.011
Nonexposed	14 (93)	1		8 (53)	7	

[1]Fisher's exact test.

TABLE VII – MULTIVARIATE LOGISTIC REGRESSION ANALYSIS OF CLINICOPATHOLOGIC CHARACTERISTICS AND EXPRESSION OF VERSICAN AND OSTEONECTIN

Variable	Hazard ratio	95% CI[1]	χ^2	p value
Sex				
Male	1	Reference	0.505	0.5
Female	0.733	0.311–1.728		
Age at diagnosis (years)				
≤65	1	Reference	1.225	0.3
>65	0.553	0.194–1.579		
T grade				
T1/2	1	Reference	0.438	0.5
T3/4	0.624	0.154–2.523		
N grade				
N0	1	Reference	2.266	0.1
N1/2/3	2.373	0.770–7.310		
Stage				
I/II	1	Reference	1.054	0.3
III/IV	2.315	0.466–11.495		
Lauren classification				
Intestinal	1	Reference	1.284	0.3
Diffuse	1.814	0.647–5.084		
Versican expression				
Diffuse positive	1	Reference	10.141	0.002
Focal positive or negative	4.484	1.783–11.364		
Osteonectin expression				
Diffuse positive	1	Reference	10.205	0.001
Focal positive or negative	6.452	2.053–20.408		

[1]Exposed *vs.* nonexposed GC.

was applied to freshly collected cancer tissue specimens from 3 survivor patients exposed to atomic-radiation as well as 20 nonexposed patients for comparison. Second, of these candidate genes, we marked out *VCAN* (encoding versican) and *SPARC* (encoding osteonectin) genes, and their protein expression was further investigated by immunohistochemical analysis with formalin-fixed and paraffin-embedded archival cancer tissue specimens from 116 survivor patients comprised of 64 exposed and 52 nonexposed cases. In our study, we found that versican and osteonectin are expressed at significantly lower levels in tumor-associated stromas of exposed patients than in nonexposed patients. Because IR is a carcinogen and can increase an individual's risk of developing cancer, analysis of early-stage GC rather than late-stage GC is important to understand the development of radiation-induced cancer. It is important to note that the clinicopathologic, phenotypic and proliferative characteristics of the early-stage (Stage I/II) GCs analyzed in our study did not differ significantly between exposed and nonexposed patients, indicating that the GC samples analyzed in our study were collected in an unbiased manner. In Stage I/II GCs, versican and osteonectin were expressed at much lower levels in tumor-associated stromas of exposed patients than in nonexposed patients. Similar results were obtained in Stage III/IV GCs. Furthermore, multivariate logistic regression analysis revealed that reduced expression of versican and osteonectin were independent markers for GCs from exposed patients. These findings suggest that tumor–stroma interaction may be altered in the development of GCs in exposed patients, typically as demonstrated by decreased expression of versican and osteonectin in tumor-associated stromas.

In our study, stromal expression of both versican and osteonectin was lower in GCs from exposed patients than in GCs from

nonexposed patients. Because transforming growth factor (TGF)-β1 can induce expression of both versican and osteonectin in cultured fibroblasts,[36,37] genes associated with TGF-β signaling pathway may be altered in exposed patients. Mice with a fibroblast-specific knockout of the TGF-β Type-II receptor rapidly develop epithelial tumors,[38] suggesting that TGF-β receptor in fibroblasts may be altered in exposed patients.

Stromal expression of both versican and osteonectin correlates with tumor progression. In our study, stromal expression of versican and osteonectin correlated with tumor stage in GC. Versican and osteonectin were expressed less frequently in stroma of exposed patients than in nonexposed patients. Phenotypic analysis revealed that although the frequency of G-type GC decreased with advancing tumor stage in nonexposed patients, the frequency of G-type GC increased with advancing tumor stage in exposed patients. In Stage III/IV GC, a marginally significant difference was observed between the frequency of G-type GC in exposed patients and that in nonexposed patients. These findings suggest the molecular mechanism of GC progression may also differ between exposed and nonexposed patients. It has been reported that tumor stage of GC is quite comparative between exposed and nonexposed patients.[39] In the present study, clinicopathologic characteristics of patients including tumor stage did not statistically differ between exposed and nonexposed patients. In addition, multivariate logistic regression analysis revealed that expression of versican and osteonectin was not significant independent marker for tumor stage. Taken together, expression of versican and osteonectin may not be involved deeply in tumor progression in GC. The biologic significance of decreased expression of versican and osteonectin in tumor progression should be investigated in detail.

Versican represses cell adhesion and promotes proliferation, migration and invasion.[10,11] Osteonectin enhances the invasive capacity of prostate and breast cancer cells.[17,18] Therefore, prognosis of exposed patients with GC may be favorable; however, it has been reported that the excess relative risk per Gy is 1.20 for mortality,[6] suggesting that stromal expression of versican or osteonectin dose not contribute significantly to aggressiveness of GC. Other molecules may be involved in poor prognosis of exposed patients with GC.

In the present study, strong and extensive staining of both versican and osteonectin were observed in tumor-associated stroma. It has been reported that both versican and osteonectin are component of the ECM. In breast cancer, the induction of versican secretion by fibroblasts isolated from normal and cancer tissues has been reported.[12] Expression of *SPARC* mRNA in liver myofibroblasts has been demonstrated by *in situ* hybridization.[40] In the present study, in addition to extracellular staining, intracellular staining of versican and osteonectin in fibroblasts were observed. Furthermore, GC cells were negative for versican and osteonectin. Taken together, these results indicate that versican and osteonectin observed in tumor-associated stroma are produced mainly by tumor-associated stromal cells.

One weak point of our study is that the A-bomb radiation doses of exposed patients with GC analyzed by oligonucleotide array were low. In addition, only 3 GCs from exposed patients were analyzed by oligonucleotide array in the present study. Analysis of additional GCs is needed. In the present study, diffuse-type GC was found more frequently in exposed patients than in nonexposed patients although the difference was not statistically significant. The diffuse-type GC contains scirrhous-type GC, which is characterized by extensive fibrous stroma, infiltrative and rapid growth and poor prognosis. Because cancer stromal cells are a mixture of fibroblasts, smooth muscle cells, endothelial cells, vascular pericytes, mesenchymal stem cells and so on, difference of cell types composing cancer stroma between A-bomb exposed and nonexposed patients should be investigated.

Although expression of *TKT*, *THBS2*, *PDGFRB* and *RNASE1* was significantly lower in GC from exposed patients than in GC from nonexposed patients by oligonucleotide array, immunoreactivities for these proteins were present in GC from exposed patients. Among 6 antibodies used in the present study, the specificity of antibodies against versican and osteonectin has been characterized in detail. In contrast, the specificity of antibodies against transketolase, THBS-2, PDGF receptor-β and ribonuclease A has not been characterized in detail. Therefore, it is possible that inconsistent results between oligonucleotide array and immunostaining represent insufficient specificity of these antibodies. Immunohistochemical analysis of these proteins by specific antibodies should be performed in the near future.

In conclusion, we found significant reduction of stromal expression of versican and osteonectin in GCs from exposed patients. Although it is unclear whether all of the GCs from exposed patients were radiation-induced cancers, versican and osteonectin may be markers for radiation-associated GC. Studies of tumor-associated stromal cells rather than tumor cells may be important to elucidate the precise long-term effects of radiation exposure.

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Immunohistochemical analysis of Reg IV in urogenital organs: Frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker

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Abstract. Regenerating islet-derived family, member 4 (Reg IV) is a candidate marker for cancer and inflammatory bowel disease and is associated with neuroendocrine and intestinal differentiation. We have reported that 14% of prostate cancer (PCa) cases are positive for Reg IV by immunohistochemistry. In the present study, we performed immunohistochemical analysis of Reg IV in other major urological cancers, including 101 renal cell carcinoma (RCC), and 95 urothelial carcinoma (UC) of urinary bladder by immunohistochemistry. We also investigated neuroendocrine differentiation by chromogranin A and synaptophysin staining along with intestinal differentiation by MUC2 staining. Immunohistochemical analysis of Reg IV revealed no expression of Reg IV in RCC, and only one case (1%) of UC expressed Reg IV. Neither neuroendocrine nor intestinal differentiation was found in RCC. Among 95 UC cases, neuroendocrine differentiation was detected in 13 cases (14%), and intestinal differentiation was observed in 33 cases (35%). In one Reg IV-positive UC case, MUC2 staining was observed. Since Reg IV expression was frequently found in PCa, we also measured Reg IV levels in sera from patients with PCa by enzyme-linked immunosorbent assay. The serum Reg IV concentration in PCa patients (n=38, mean ± SE, 1.69±0.16 ng/ml) was significantly higher than that in control individuals (n=40, 1.28±0.11 ng/ml, P=0.0199, Mann-Whitney U test). The sensitivity and specificity for detection of PCa were 34% (13/38) and 90% (36/40), respectively. These results suggest that among major urologic cancers, Reg IV is expressed frequently in PCa, and that serum Reg IV represents a novel biomarker for PCa.

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Introduction

Prostate cancer (PCa) is one of the most common types of cancer and is the second leading cause of cancer death among men in the United States (1). Currently, the standard diagnostic marker for PCa is prostate-specific antigen (PSA) and the rapid incorporation of aggressive PSA testing has resulted in dramatically earlier identification of PCa (2). However, the significantly high false-positive rate of PSA combined with its widespread clinical application, has lead to a tremendous increase in the number of unnecessary prostate biopsies (3). Therefore, there has been an increasing emphasis in the need to determine multiple protein biomarkers for use in the diagnosis and prognosis of PCa.

Renal cell carcinoma (RCC) accounts for ~2% of all human cancers worldwide, and in 2000 189,000 cases and 91,000 deaths were reported (4). RCC is characterized by an absence of early warning signs leading to a rather high percentage of advanced, metastatic tumors upon diagnosis. For example, the classic triad of pain, hematuria, and a palpable flank mass is encountered in only 10% of patients and is usually associated with the presence of advanced disease (5). Unlike other solid malignancies in which established serum or urinary biomarkers are available for early detection, relatively few diagnostic tools are currently available for the early detection of renal tumors. Therefore, an ideal tumor marker with high sensitivity and specificity offers the ideal opportunity for early detection of RCC.

Bladder cancer, the majority of which is urothelial carcinoma (UC) is the sixth most common cancer in the world. A distinctive feature of UC is that multiple metachronous or synchronous cancers frequently develop, arising from either a polyclonal origin or metastasis from a single clone. Thus, bladder cancer patients need a long-term follow-up with repeated urine cytology and cystoscopy for monitoring. Conventional urine cytology has been the standard non-invasive method for cancer detection and disease monitoring.

However, the sensitivity of this method is known to be low, especially for low-grade UC. Therefore, a more sensitive, non-invasive method for cancer detection is required.

We previously performed serial analysis of gene expression (SAGE) of four primary gastric cancers (6) and identified several gastric cancer-specific genes (7). Of these genes, regenerating islet-derived family, member 4 (REG4, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with gastric cancer. REG4 is a member of the REG gene family, which includes three other genes, and was originally identified by high-throughput sequence analysis of a large inflammatory bowel disease cDNA library (8). Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that ~50% of gastric cancers overexpress the REG4 gene (6) and Reg IV expression is associated with the intestinal and neuroendocrine differentiation (9). In addition to gastric cancer, overexpression of Reg IV in colorectal cancer (10), pancreatic cancer (11) and PCa (12) has been reported. In our previous immunohistochemical analysis (13), 14% of the PCa cases were positive for Reg IV and Reg IV positivity was associated with intestinal and neuroendocrine differentiation. Furthermore, Reg IV expression is an independent prognostic indicator of relapse after radical prostatectomy. However, histologically, gastric, colorectal, pancreatic and prostate cancers are adenocarcinomas, and expression of Reg IV except for adenocarcinomas, such as RCC or UC, has not yet been investigated.

Reg IV is a secreted protein which we have previously shown represents a novel biomarker for gastric cancer (14). The diagnostic sensitivity of serum Reg IV was superior to that of serum carcinoembryonic antigen or carbohydrate antigen 19-9. Serum Reg IV serves as a tumor marker for colorectal and pancreatic cancer (10,11). These data support the idea that Reg IV protein has potential as a novel serum tumor marker for a wide variety of malignancies; however, serum concentration of Reg IV in major urologic cancers including PCa, RCC or UC has not previously been measured.

In the present study, we examined the expression and distribution of Reg IV in human RCC and UC by immunohistochemistry. We have reported two Reg IV staining patterns (mucin-like and perinuclear staining) (9). Mucin-like Reg IV staining, observed in goblet cells and goblet cell-like vesicles of tumor cells, is associated with MUC2 (a goblet cell marker) positivity. Perinuclear Reg IV staining is detected in cells with neuroendocrine differentiation. Therefore, we also performed immunohistochemical analysis of MUC2, chromogranin A (a neuroendocrine cell marker) and synaptophysin (a neuroendocrine cell marker). Since Reg IV expression was frequently found in PCa, we also measured Reg IV levels in sera from patients with PCa by enzyme-linked immunosorbent assay (ELISA) to investigate the potential diagnostic utility of Reg IV measurement.

Materials and methods

Tissue samples. In total, 204 primary tumor samples and 78 serum samples were collected. Patients were treated at the Hiroshima University Hospital or an affiliated hospital.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 196 patients who had undergone surgical excision for either RCC (n=101) or UC (n=95). All 101 patients with RCC were treated by radical nephrectomy, and all 95 patients with UC were treated by cystectomy. Tumor staging was performed according to the TNM classification system (15).

For Western blot analysis, 8 RCC samples and the corresponding non-neoplastic kidney samples were used. The samples were obtained during surgery at Hiroshima University Hospital or an affiliated hospital. We confirmed microscopically that the tumor specimens were predominantly RCC tissue (>80%). Samples were frozen immediately in liquid nitrogen and stored at -80˚C until use.

All serum samples were collected before prostate biopsy and stored at -80˚C. Seventy-eight consecutive men who visited the outpatient clinic of the Department of Urology, Hiroshima University Hospital due to elevated PSA levels (>4.0 ng/ml) served as the study population. The PCa population consisted of 38 men with evidence of cancer from a prostate biopsy (age range 50-93 years, mean 73 years), and the remaining 40 men without evidence of cancer from a prostate biopsy served as a control population (age range 47-90 years, mean 66 years). In accordance with the Ethics Guidelines for Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used on the basis of approval from the Ethics Review Committee of the Hiroshima University School of Medicine and from the ethics review committees of collaborating organizations.

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immunohistochemically stained. Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min for Reg IV, MUC2, chromogranin A and synaptophysin. Peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min and sections were then incubated with normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min to block non-specific antibody binding. Sections were incubated with a primary antibody against Reg IV (rabbit polyclonal antibody, diluted 1:50; anti-Reg IV antibody was raised and characterized in our laboratory) (9), MUC2 (1:50; Novocastra, Newcastle, UK), chromogranin A (1:50; Novocastra), or synaptophysin (1:50; Dako Cytomation) for 1 h at room temperature, followed by incubation with peroxidase-labeled anti-rabbit or anti-mouse IgG for 1 h. Staining was completed with a 10-min incubation in a substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The specificity of the Reg IV antibody has been characterized in detail (9). Staining of each antibody was considered positive if any tumor cells were stained. Intestinal differentiation was defined as positive staining for MUC2. Neuroendocrine differentiation was defined as positive staining for chromogranin A and/or synaptophysin.

Western blot analysis. Preparation of whole cell lysates from the RCC samples and Western blotting were performed as previously described (16). Protein concentrations were determined by Bradford protein assay (Bio-Rad, Richmond,

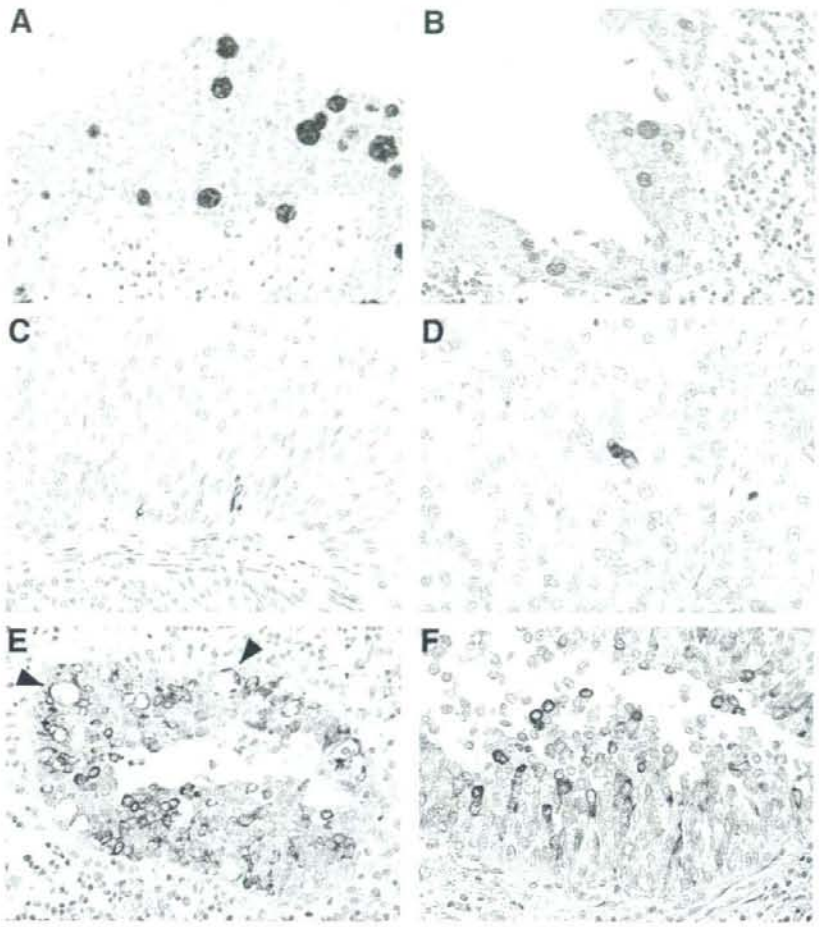

Figure 1. Immunohistochemical analysis of Reg IV expression in UC of the urinary bladder. (A) Immunostaining of Reg IV in UC. In case 37, mucin-like staining of Reg IV is present in goblet cell-like vesicles of tumor cells. (B) In case 37, PAS staining was observed in gland-like lumina. (C) Immunostaining of chromogranin A in UC. (D) Immunostaining of synaptophysin in UC. (E) Immunostaining of MUC2 in UC. In case 37, mucin-like and perinuclear staining are observed in tumor cells. Arrowhead indicates mucin-like staining of MUC2. (F) Immunostaining of MUC2 in UC. Perinuclear staining of MUC2 is present in tumor cells.

CA, USA) with BSA used as the standard. Protein (20 μg per lane) was electrophoresed on SDS-PAGE gels and transferred to nitrocellulose filters. Filters were incubated for 1 h at room temperature with anti-Reg IV antibody (rabbit polyclonal antibody raised in our laboratory). Peroxidase conjugated anti-rabbit IgG was used in the secondary reaction. The immunocomplexes were visualized with an ECL Western blot detection system (Amersham Biosciences, Piscataway, NJ, USA). The quality and amounts of proteins on the gel were confirmed by detection with anti-ß-actin antibody (Sigma-Aldrich, St. Louis, MO, USA).

ELISA. For measurement of the serum Reg IV concentration, a sandwich ELISA method was developed as previously described (14). First, polystyrene microtiter plates were coated with mouse monoclonal anti-Reg IV antibody (R&D Systems, Abingdon, UK) by overnight incubation of 50 μl/125 ng per well of antibody diluted in Tris buffer (pH 7.4). The plates were then washed 3 times with wash buffer. After the plates were blocked with 1% milk in phosphate-buffered saline, 50 μl of recombinant Reg IV standard or sample was added to each well and incubated overnight at 4˚C. After 3 washes, 50 μl of biotinylated goat polyclonal anti-Reg IV antibody (R&D Systems) in assay buffer [1% BSA, Tris buffer (pH 7.4) and 0.05% normal goat serum] was added to each well (75 ng antibody per well). The mixture was then incubated for 1 h with shaking at 37˚C and washed 3 times with wash buffer. The plates were incubated with 50 μl per well alkaline phosphatase-conjugated streptavidin (Dako) diluted 1:2000 in diluent containing 1% BSA and Tris buffer (pH 7.4) for 1 h at 37˚C and washed 3 times. Color development was performed with the addition of pNPP chromogenic substrate (Sigma-Aldrich) followed by incubation at 37˚C for 1 h. Absorbance at 405 nm was measured with an ELISA

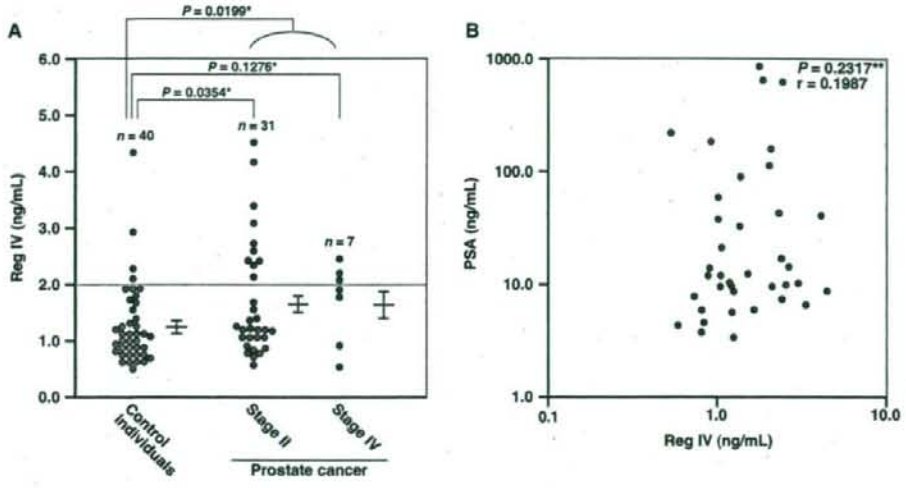

Figure 2. ELISA of serum samples from 40 control individuals and 38 patients with PCa. (A) Detection of Reg IV protein in serum samples by ELISA. Yellow bars indicate the cut-off levels defined on the basis of the previous study [2.00 ng/ml (14)]. Red bars indicate the mean ± SE. Differences in the serum concentration of Reg IV between two groups are tested by Mann-Whitney U test (*). (B) Relation between serum concentrations of Reg IV and PSA. Correlation is examined using Spearman's rank correlation (**).

plate reader. As a reference standard, known concentrations of human recombinant Reg IV (9) from 0 to 30 ng/ml were tested in triplicate.

Statistical methods. Association between clinicopathologic variables and Reg IV expression was analyzed by Fisher's exact test. Differences in the serum Reg IV concentration between the two groups were tested by the non-parametric Mann-Whitney U test. Correlations between the serum Reg IV concentration and the serum concentration of PSA were assessed by Spearman's rank correlation test. $P<0.05$ was considered statistically significant.

Results

Expression of Reg IV and neuroendocrine and intestinal differentiation in RCC. We performed immunohistochemical analysis of Reg IV in 101 RCC samples; however, no staining of Reg IV was found. In adjacent non-neoplastic kidney tissues, no Reg IV staining was found. Western blot analysis of Reg IV was also performed in 8 RCC samples (data not shown), in which no Reg IV expression was confirmed in the RCC samples nor in adjacent non-neoplastic kidney samples. We also performed immunohistochemical analysis of chromogranin A, synaptophysin and MUC2; however, no staining was detected.

Expression of Reg IV and neuroendocrine and intestinal differentiation in UC. We performed immunohistochemical analysis of Reg IV in 95 UC samples. In adjacent non-neoplastic urinary bladder tissues, no Reg IV staining was found. In UC tissues, Reg IV staining was observed in 1 (1%) of 95 UC cases. In this Reg IV-positive UC case (case 37), Reg IV-positive tumor cells were observed in 10% of tumor cells, and mucin-like staining (Fig. 1A) was observed. In case 37, Reg IV staining was observed in gland like lumina, and periodic acid-Schiff (PAS) staining was also observed in gland-like lumina (Fig. 1B), indicating that this Reg IV-positive case represents glandular differentiation. Analysis of the relationship between Reg IV staining and clinicopathologic characteristics showed that Reg IV did not correlate with gender, age, pT category, pN category or tumor stage (data not shown).

Immunostaining of chromogranin A and synaptophysin was also performed. Representative results of chromogranin A and synaptophysin immunostaining in UC are shown in Fig. 1C and D, respectively. Of the 95 UC cases, chromo-granin A-positive cases were observed in 3 cases (3%) and synaptophysin-positive cases were found in 11 cases (12%). In total, UC cases showing neuroendocrine differentiation were found in 13 cases (14%). However, case 37 (Reg IV-positive UC case) did not show neuroendocrine differentiation. Analysis of the relationship between neuroendocrine differentiation and clinicopathologic characteristics showed no correlation between neuroendocrine differentiation and gender, age, pT category, pN category or tumor stage (data not shown).

In UC tissues, MUC2 staining was observed in goblet cell-like vesicles (Fig. 1E) and perinuclear regions (Fig. 1F) of tumor cells. Of the 95 UC cases, MUC2-positive cases were observed in 33 cases (35%). Among the 33 UC cases showing positive results for MUC2, one case, case 37 (Reg IV-positive UC case), showed MUC2 staining in goblet cell-like vesicles and perinuclear regions. The remaining 32 cases showed only perinuclear MUC2 staining. Analysis of the relationship between MUC2 positivity and clinicopathologic characteristics showed no correlation between MUC2-positivity and gender, age, pT category, pN category or tumor stage (data not shown).

Serum Reg IV concentration in patients with PCa. As Reg IV expression was frequently found in PCa, we also measured

Reg IV levels in sera from patients with PCa by ELISA to investigate the potential diagnostic utility of Reg IV measurement. Serum Reg IV levels in 40 control individuals and 38 patients with PCa prior to biopsy are shown in Fig. 2A. The serum Reg IV concentration in PCa patients (n=38, 1.69±0.16 ng/ml, mean ± SE) was significantly higher than that in control individuals (n=40, 1.28±0.11 ng/ml, P=0.0199, Mann-Whitney U test), even at stage II (n=31, 1.69±0.18 ng/ml, P=0.0354, Mann-Whitney U test) (Fig. 2A). The mean of serum Reg IV concentration in PCa patients at stage IV (n=7, 1.68±0.26 ng/ml) was higher than that in control individuals (n=40, 1.28±0.11 ng/ml); however, statistical difference was not found (P=0.1276, Mann-Whitney U test). The Reg IV concentration in serum samples from patients with PCa showing a Gleason score of ≤7 (n=22, 1.75±0.21 ng/ml) was not significantly different from those showing a Gleason score of ≥8 (n=16, 1.60±0.24 ng/ml) (P=0.7448, Mann-Whitney U test). In our previous study, the cut-off level for Reg IV was set at 2.00 ng/ml (14). In the group showing serum PSA levels of >4 ng/ml, the sensitivity and specificity for detection of PCa were 34 (13/38) and 90% (36/40), respectively. Spearman's rank correlation test revealed that significant correlation was not found between serum Reg IV and PSA levels (r=0.1987, P=0.2317) (Fig. 2B).

Discussion

Previously, we showed that 14% of the PCa cases were positive for Reg IV, and Reg IV positivity was associated with intestinal and neuroendocrine differentiation (13). These PCa cases were all adenocarcinomas. In the present study, immunohistochemical analysis of Reg IV was performed in other major urologic cancers including RCC and UC, both of which are not adenocarcinomas. Immunohistochemical analysis of Reg IV revealed that Reg IV expression was not found in RCC, and only 1% of UC expressed Reg IV. These results and our previous immunohistochemical analysis of Reg IV in PCa indicate that Reg IV is expressed frequently in PCa among major urologic cancers. Furthermore, we showed that the serum Reg IV concentration in PCa patients was significantly higher than that in control individuals. The sensitivity of serum Reg IV concentration was 34%, and the specificity was 90%. It has been reported that the specificity of the PSA test is only 20% at a sensitivity of 80% (17), indicating that serum Reg IV concentration serve as a diagnostic tumor marker with high specificity. Since serum Reg IV concentration was measured in the group showing serum PSA levels of >4 ng/ml, the sensitivity and specificity calculated in the present study may differ from those in healthy individuals, and serum concentration of Reg IV should also be measured in the group showing serum PSA levels of <4 ng/ml.

In urologic cancers including PCa, RCC and UC, urine represents a particularly useful fluid in which to examine tumor markers because of its enhanced potential to contain higher concentrations of directly released tumor-derived products and the fact that collection is non-invasive. In the present study, Reg IV was detected in serum samples from patients with PCa. Reg IV expression was not found in RCC, and only 1% of UC expressed Reg IV. In adjacent non-neoplastic kidney and urinary bladder tissues, no Reg IV staining was found. These results suggest that Reg IV concentration in urine may represent a novel marker for PCa at high specificity.

In RCC, no Reg IV expression was detected. Although neuron-specific enolase (a neuroendocrine cell marker) is frequently expressed in RCC, chromogranin A expression is rare (18). It has been reported that staining of MUC2 is not found in 16 RCC cases (19). In the present study, we also observed that RCC showed neither neuroendocrine differentiation based on chromogranin A and synaptophysin staining nor MUC2 staining. As Reg IV expression is associated with neuroendocrine and intestinal differentiation, these results are accordance with immunonegativity of Reg IV in RCC.

In UC, only 1 (1%) UC case showed Reg IV staining. In the Reg IV-positive UC case, Reg IV staining was observed in gland-like lumina. Although this case was UC, PAS staining was also observed in gland-like lumina, indicating that this Reg IV-positive case possessed glandular differentiation. These results indicate that conventional UC is negative for Reg IV; however, UC with glandular differentiation may express Reg IV. A previous immunohistochemical study demonstrated that expression of chromogranin A is seen in 63.5% of UC cases (20). Yet, in the present study, only 14% of UC cases showed neuroendocrine differentiation and did not exhibit Reg IV expression. However, in gastric cancer, chromogranin A-positive tumor cells are not always positive for Reg IV (9). Furthermore, the one UC case with glandular differentiation was positive for MUC2 in goblet cell-like vesicles. Yet, Reg IV staining was not found in UC cases showing perinuclear MUC2 staining, nor was glandular differentiation observed in these cases. Based on this evidence, Reg IV expression is not likely involved in neuroendocrine differentiation of UC but is likely associated with glandular differentiation.

In conclusion, we showed that serum Reg IV concentration serves as a diagnostic tumor marker with high specificity for PCa. As the significantly high false-positive rate of PSA has lead to a tremendous increase in the number of unnecessary biopsies of the prostate (3), measurement of serum concentration of Reg IV may decrease the number of unnecessary prostate biopsies. Reg IV is correlated with relapse-free survival of patients with PCa (13), and hormone-refractory PCa has been reported to express high levels of REG4 mRNA (12). Therefore, serum concentration of Reg IV may predict relapse-free survival and resistance to androgen-deprivation therapy.

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Reg IV enhances peritoneal metastasis in gastric carcinomas

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Abstract

Objectives: The role of Regenerating (Reg) IV on peritoneal metastasis was examined in gastric cancer using.

Material and methods: *Reg IV*-transfected human gastric cancer cells (MKN28-R1, MKN28-R2, TMK1-R1), control transfectants (MKN28-R0, TMK1-R0), and *REG4*-knocked down MKN45 cells were examined in *in vitro* and in nude mice peritoneal metastasis models.

Results and Discussion: Increase of expression and secretion of Reg IV, and levels of BCL-2, BCL-XL, survivin, phosphorylated AKT, and phosphorylated EGFR, and decrease of nitric oxide-induced apoptosis were found in *Reg IV*-transfectants, whereas those were abrogated in the knockdown cells. In mice models, increased number and size of peritoneal tumors and decreased apoptosis were found in *Reg IV*-transfectants, whereas those were abrogated by the knockdown cells. Mice survivals were worsened in *Reg IV*-transfectants-inoculated mice, but were improved in *Reg IV*-knockdown cell-inoculated mice. Levels of Reg IV protein in peritoneal lavage fluids increased in *Reg IV*-transfectants inoculated mice, but decreased in *Reg IV*-knockdown cell inoculated mice. In metastasized human gastric cancers, Reg IV positivity in peritoneum-metastasis cases was higher than those in negative cases. Reg IV was detected in peritoneal lavage fluids from human gastric cancer patients, in whose lavages keratin mRNA was detected by reverse transcriptase-polymerase chain reaction. Collectively, Reg IV might accelerate peritoneal metastasis in gastric cancer. Reg IV in lavage fluids might be a good marker for peritoneal metastasis.

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Introduction

Gastric cancer is a leading cause of cancer death in the world, and is the second leading cause of deaths from cancer in Japan (1,2). Approximately 20% of gastric cancer patients show peritoneal and/or liver metastases at surgery (3), and 30% of who have died from gastric cancer suffered from peritoneal metastasis (4). Peritoneal metastasis causes the terminal stage of advanced gastric cancer, and diminishes the quality of patients' life by intestinal obstruction, ascites retention and subsequent malnutrition. Control of peritoneal metastasis is expected to improve patients' quality of life (5,6).

The molecular mechanism of peritoneal metastasis is an ongoing assignment of cancer research. We have identified loss of heterozygosity of chromosome 7q involving 7q35 locus as a peritoneal metastasis-associated event in gastric cancer (7). Overexpression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukin-8, is associated with peritoneal metastasis and ascites production in ovarian cancer (8). The truncated form of fibroblast growth factor/keratinocyte growth factor receptor 2 IIIb (K-*sam*) and c-*met* show gene amplification and/or overexpression in scirrhous-type gastric cancers, which frequently produces peritoneal metastasis (9–11). Gene expression profiling shows alteration in expression of several genes, such as up-regulation of trefoil factor 1, α-1-antitrypsin and galectin 4, and down-regulation of cytidine deaminase (12). Recently, we reported the importance of activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) in suppressing peritoneal metastasis, which provides tumour growth inhibition and apoptosis induction in gastric and colon cancer cells (13,14). The importance of survival factors in peritoneal metastasis formation is emphasized in many reports; RUNX3, survivin, nuclear factor κB and Bcl-2/Bag are also associated with peritoneal metastasis (15–18).

The *Reg* (*regenerating*) gene family belongs to the calcium-dependent lectin superfamily (19,20). *Reg IV* is a new member family, and it is identified as a gene expressed in the gastrointestinal tract and pancreas (21,22). Human

Reg IV gene is located on chromosome 1, unlike other *Reg* family genes, which are located on 2p12 (23). *Reg IV* is expressed in Crohn's disease and ulcerative colitis (21,24) and is revealed to be associated with malignant potential of colorectal adenocarcinomas and malignant transformation of colorectal adenomas (25,26). Recently, Reg IV has been reported to activate epidermal growth factor receptor (EGFR), protein kinase B/Akt and activator protein-1 to accelerate colorectal cancer cell survival by increasing Bcl-2, Bcl-XL and survivin (27). The anti-apoptotic property of Reg IV is associated with colorectal cancer development and drug resistance in gastric cancer (28,29) and its expression is expected to be a marker for highly malignant potential (30–32).

We have identified *Reg IV* as a cancer-affiliated expressed gene, by a serial analysis of gene expression (SAGE) technique, in which results were deposited in the NCBI SAGE Library in the Web (http://www.ncbi.nlm.nih.gov/SAGE/). Reg IV protein is immunohistochemically detected in 36% of colorectal adenocarcinomas and this is associated with tumour stage, whereas Reg IV production is detected in 29% of gastric adenocarcinomas, and is associated with both the intestinal mucin phenotype and neuroendocrine differentiation but not with tumour stage or patient prognosis (33). Thus, the role of Reg IV in gastric cancer is still unclear.

In this study, we attempted to determine the relevance of Reg IV expression in peritoneal metastasis of gastric cancer using *Reg IV*-transfected human gastric cancer cells.

Materials and Methods

Cell culture

Human gastric cancer cell lines, MKN28 and TMK1, were transfected with the *Reg IV* expression vector (29,33). MKN28- and TMK1-*Reg IV* stable transfectants were selected by G418 (Sigma Chemical Co., St. Louis, MO, USA). Three transfectants with marked *Reg IV* expression were used in the present study, which were designated as MKN28-R1, MKN28-R2 and TMK1-R1. For the control, MKN28 and TMK1 cells were transfected with empty vector, which was designated as MKN28-R0 and TMK1-R0. MKN45 cells, which express Reg IV at a high level, was treated with *Reg IV* siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or mixed siRNA for control. siRNA (50 nM for 2×10^5 cells) diluted with transfection solution (Santa Cruz Biotechnology) was used for treatment according to the manufacturer's instructions. The cells were routinely maintained in RPMI-1640 (Sigma Chemical) containing 10% foetal bovine serum (Sigma Chemical) and G418 (500 μg/ml) at 37 °C in a 5% CO_2/95% air atmosphere.

Tissue samples

Gastric cancer cases with metastasis were chosen from the patients operated on in Hiroshima University Hospital, Nara Medical University Hospital, and Miyoshi Central Hospital. Among the 85 cases, all showed lymph node metastasis, 8 cases showed liver metastasis, and 43 cases showed peritoneal metastasis at surgery. Of 42 cases without peritoneal metastasis (found via gross and cytological examinations) at the operation, 21 cases showed peritoneal recurrence. For immunohistochemistry, representative formalin-fixed, paraffin-embedded tissue samples were used, which contained the deepest invasive portion of the tumour.

Cell population growth

The cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% ethylenediaminetetraacetic acid (Sigma Chemical). Cells were seeded at a density of 10 000 cells per well in 24-well tissue culture plates and treated under the conditions mentioned in the Results section. Cell number was counted using an autocytometer (Sysmecs, Kobe, Japan) at 24, 48 and 72 h. The experiment was repeated three times. For sodium nitropusside (SNP) treatment, cell number was counted 48 h after the treatment.

In vitro *invasion assay*

A modified Boyden chamber assay was performed to examine *in vitro* invasion of MKN28 cells. Polycarbonate filters (pore size 3 μm; diameter 5 mm) were glued to collagen type IV inserts (Becton-Dickinson Labware, Bedford, MA, USA), which were placed in the wells of 24-well tissue culture plates. The cells were suspended in 500 μl of regular medium and placed in the upper part of the chamber. The lower part of the chamber was filled with regular medium. After 24-h incubation at 37 °C, filters were carefully removed from the inserts, stained with haematoxylin for 10 min and mounted on microscope slides. The number of stained cells was counted in whole inserts wide at ×100 magnification. Invasion activity was quantified by average number of cells per insert well. Mean values of invading cells were calculated from the results of three independent experiments.

Animal model

BALB/c nu-nu athymic mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mice were maintained according to the institutional guidelines approved by the Committee for Animal Experimentation of the Nara Medical University, in accordance with the current regulations and standards

of the Ministry of Health, Labour and Welfare. The mice were used according to the institutional guidelines when they were 5 weeks old. Cells (as above) were briefly trypsinized and washed with Hanks' balanced saline solution (HBSS) three times. They were suspended in HBSS and were injected into the peritoneal cavity (1×10^7) of each mouse; eight or nine mice were injected per group. The mice were sacrificed to count numbers of metastatic foci in the peritoneal cavity. In another experimental set, survival of 10 mice was observed in each cell line until 16 weeks after the inoculation. Mice inoculated with MKN45 cells treated with siRNA were administrated siRNA encapsulated with liposome (34). siRNA (100 pmol) was encapsulated with 2 ml of kationic liposome (EL-C-01, Nippon-Oil_Fats Co., Tokyo, Japan), and 200 µl of the solution was administrated intraperitoneally to each mouse twice a week.

Immunohistochemistry

Consecutive 4-µm sections were cut from each block, and immunostaining was performed by immunoperoxidase technique following antigen retrieval with citrate buffer (pH 6.0) treatment for 10 min (three times). After endogenous peroxidase block by 3% hydrogen peroxide–methanol for 15 min, specimens were rinsed with 5% washing solution (BioGenex, San Ramon, CA, USA). Anti-Reg IV antibody established in our laboratory was used at 0.5 µg/ml primary antibody, and incubated at room temperature for 2 h (33). Specimens were rinsed with 5% washing solution and incubated at room temperature for 1 h with secondary antibody conjugated to peroxidase diluted at 0.5 µg/ml (anti-rabbit IgG, Medical & Biotechnological Laboratories Co., Ltd, Nagoya, Japan). All specimens were then rinsed with 5% washing solution and colour was developed by diaminobenzidine solution (Dako, Glostrup, Denmark). After washing with water, specimens were counterstained with Meyer's haematoxylin (Sigma Chemical). Immunostaining of all specimens was performed to ensure the same condition of antibody reaction and diaminobenzidine exposure.

Preparation of conditioned medium, peritoneal lavage and serum

Cells were cultured in RPMI-1640 containing 1% foetal bovine serum for 12 h. Then, the conditioned medium was filtered with 0.2-µm filter (Becton-Dickinson Labware). The peritoneal cavity of a sacrificed mouse was washed with 2 ml of phosphate-buffered saline and saved phosphate-buffered saline was filtered with 0.2-µm filter (Becton-Dickinson Labware). Mouse blood was obtained by cardiac puncture, mixed with heparin (5% v/v), and centrifuged at 500 ***g*** for 15 min at 4 °C. The supernatant serum was used for immunoblot analysis. For human peritoneal lavage, the entire peritoneal cavity was washed with 100 ml saline after opening of the peritoneal cavity. Of the peritoneal lavage fluid, 10 ml was centrifuged at 500 ***g*** for 15 min at 4 °C. The supernatant was used for immunoblotting and the pellet was used for reverse transcriptase–polymerase chain reaction (RT-PCR). Remnants of lavage fluid were used for cytological examination. Slot blotted lavage fluids and cultured media stained with Coomassie blue dye were served as the control for the sample loading.

Immunoblot analysis

Whole-cell lysates were prepared as described previously (35). The cultured medium and peritoneal lavage fluids were concentrated with the Protein Concentrate kit (TaKaRa Bio Inc., Shiga, Japan). Forty-microgram lysates were subjected to immunoblot analysis in 12.5% sodium dodecyl sulphate–polyacrylamide gels followed by electrotransfer on to nitrocellulose filters. Filters were incubated with primary antibodies and then with peroxidase-conjugated IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan). α-tubulin or β-actin antibodies assessed levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA, USA). The immune complex was visualized by CSA system (Dako) or ECL system (Amersham Biosciences Corp., Piscataway, NJ, USA). Antibodies for Reg IV (33), Bcl-2 (Dako), Bcl-XL, survivin (Santa Cruz Biotechnology), phosphorylated AKT (phospho-Ser473, Upstate Biotechnology Inc., Lake Placid, NY, USA), phosphorylated EGFR (phospho-Tyr992, Cell Signaling Technology, Beverly, MA, USA), and EGFR (Cell Signaling Technology) were used as primary antibodies.

Detection of cytokeratin in peritoneal lavage

Pellet from the peritoneal lavage fluids were used for RT-PCR, which was performed with iScript One-Step RT-PCR Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers for cytokeratin 20 mRNA were 5′-GAG GTT CAA CTA ACG GAG CT-3′ (forward) and 5′-TCT CTC TTC CAG GGT GCT TA-3′ (reverse) were referred to GenBank NM019010, which were synthesized by Sigma Genosys (Ishikari, Japan).

Statistical analysis

Statistical significance was examined by two-tailed Fisher's exact test, two-tailed *chi*-squared test, and two-tailed, unpaired Mann–Whitney test by using InStat software (GraphPad Software, Los Angels, CA, USA). Survival

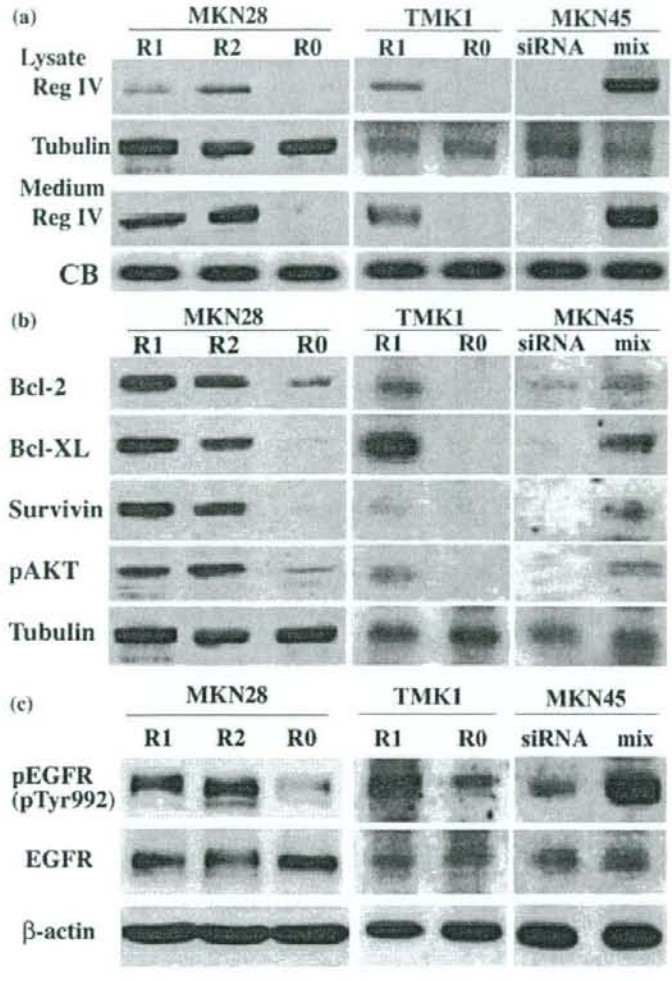

Figure 1. Production and secretion of Reg IV and survival-related proteins in gastric cancer cells. (a) Production and secretion of Reg IV protein were examined in lysates and culture media by immunoblotting. Tubulin and total loading protein detected by Coomassie blue staining (CB) served as loading controls. (b) Production of cell survival-related factors (Bcl-2, Bcl-XL, survivin and phophorylated AKT) was examined by immunoblotting. Tubulin served as loading control. (c) Phosphorylation levels of Tyr992 of EGFR were examined by immunoblotting. (a–c) MKN28-R1, MKN28-R2 and TMK1-R1: stable *Reg IV* transfectants. MKN28-R0 and TMK1-R0: empty vector-transfectant. MKN45-siRNA: *Reg IV* siRNA-treated MKN45 cells. MKN45-mix: siRNA mixture-treated MKN45 cells. β-actin or tubulin served as loading controls.

curves were calculated by Kaplan–Meier model (Statview 4.5, Abacus Concepts Inc., Berkeley, CA, USA). Difference of survivals was calculated by Cox proportional hazard model (Statview 4.5). Statistical significance was defined as a two-sided *P*-value of less than 0.05.

Results

Production of Reg IV protein and survival factors in gastric cancer cells

We first confirmed expression of Reg IV protein in *Reg IV*-transfected MKN28 and TMK1 cells and Reg IV siRNA-treated MKN45 cells (Fig. 1a). Three *Reg IV*-transfected cells (MKN28-R1, MKN28-R2 and TMK1-R1) and control vector-transfected cells (MKN28-R0 and TMK1-R0) were examined to detect Reg IV protein in the cell lysates and cultured media. MKN28-R0 and TMK1-R0 cells showed undetectable levels of Reg IV in the lysate and cultured media, whereas MKN28-R1, MKN28-R2 and TMK1-R1 cells produced Reg IV in the lysates and media. siRNA mixture-treated MKN45 cells expressed Reg IV at high level, whereas Reg IV siRNA-treated MKN45 cells did not express Reg IV. Second, levels of survival-related proteins in these cells were examined (Fig. 1b). Protein levels of Bcl-2, Bcl-XL, survivin and phorphorylated

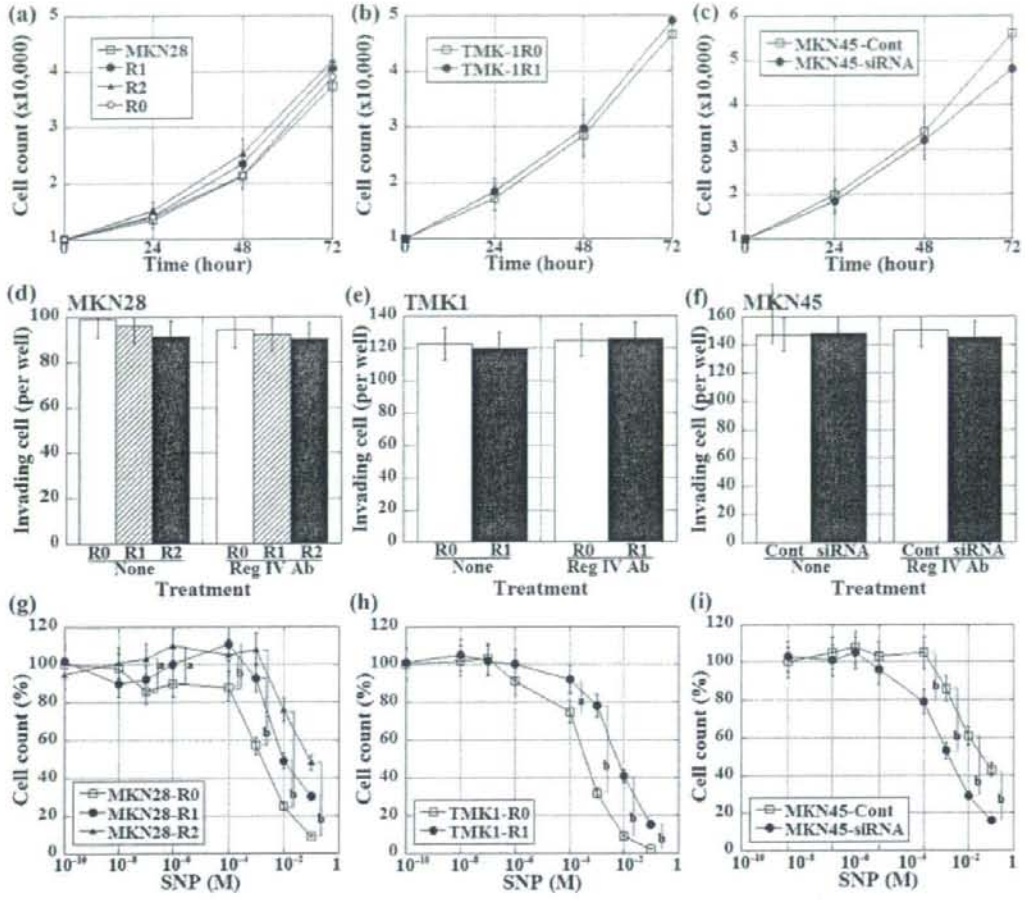

Figure 2. Effects of Reg IV transfection on cell population growth, *in vitro* invasion and nitric oxide-induced apoptosis in gastric cancer cells. (a–c) Cell growth of Reg IV transfectants and Reg IV siRNA-treated cells. (d–f) Invasion activity was examined by *in vitro* invasion assay with type IV collagen-coated insert. Anti-Reg IV polyclonal antibody was added to culture media for neutralizing secreted Reg IV at 0.5% v/v ('Reg IV Ab'). (g–i) Sensitivity to nitric oxide-induced cytotoxicity was examined. Sodium nitroprusside (SNP) was used as nitric oxide donor. (a) $P < 0.001$; (b) $P < 0.0001$. (a–c) MKN-R1, MKN-R2 and TMK1-R1: stable *Reg IV*-transfectants. MKN28-R0 and TMK1-R0: empty vector-transfectant. MKN45-siRNA: *Reg IV* siRNA-treated MKN45 cells. MKN45-Cont: siRNA mixture-treated MKN45 cells. Error bar: standard deviation.

AKT in MKN28-R1, MKN28-R2, TMK1-R1 and siRNA mixture-treated MKN45 cells were higher than those in MKN28-R0, TMK1-R0 and Reg IV siRNA-treated MKN45 cells. Third, phosphorylated form of EGFR (phosphotyrosin 992) in these cells was examined (Fig. 1c). pEGFR levels in MKN28-R1, MKN28-R2, TMK1-R1 and siRNA mixture-treated MKN45 cells were higher than those in MKN28-R0, TMK1-R0 and Reg IV siRNA-treated MKN45 cells.

Effect of Reg IV transfection on cell population growth, invasion and survival of gastric cancer cells

Next, biological effects of *Reg IV* transfection on MKN28 cells were examined (Fig. 2). As shown in Fig. 2(a), growth of MKN28-R1 and MKN28-R2 cells was not different from MKN28-R0 and MKN28 parental cells. Numbers of MKN28-R1 and MKN28-R2 cells invading into type IV collagen-coated membranes was not different

